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<b>(21) International Application Number:</b> PCT/US91/03082 <b>(22) International Filing Date:</b> 3 May 1991 (03.05.91) <b>(30) Priority data:</b> 521,576 10 May 1990 (10.05.90) US <b>(71) Applicant:</b> THE DANA-FARBER CANCER INSTITUTE [US/US]; 44 Binney Street, Boston, MA 02115 (US). <b>(72) Inventor:</b> ROCK, Kenneth, L. ; 59 Dwinell Street, West Roxbury, MA 02132 (US). <b>(74) Agent:</b> HART, Julia, D.; Kramer, Brufsky & Cifelli, 181 Old Post Road, P.O. Box 59, Southport, CT 06490 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), BR, CH (European patent), DE (European patent), DK (European patent), ES, ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> ENHANCING THE ASSOCIATION OF EXOGENOUS PEPTIDES WITH CLASS I MHC MOLECULES ON IMMUNE SYSTEM CELLS  <b>(57) Abstract</b> <p>The present invention provides a method for enhancing the association of exogenously prepared polypeptides with class I MHC molecules on the surface of antigen presenting cells to thereby sensitize the target cells for class I MHC-restricted cytotoxic T cell response. The method involves obtaining the polypeptides of interest and presenting the polypeptides to the appropriate antigen presenting cells in the presence of an elevated level of free <math>\beta_2</math>-microglobulin. The method of the present invention can also be used to prime naive populations of T lymphocytes for antigen-specific class I MHC-restricted cytotoxic T cell response with exogenous polypeptide and is useful in both <i>in vitro</i> and <i>in vivo</i> applications. A new use for purified <math>\beta_2</math>-microglobulin as an adjuvant for synthetic polypeptide vaccines is also provided. <i>In vivo</i> immunization with the synthetic peptides and free <math>\beta_2</math>-microglobulin can elicit an antigen-specific CTL response as strong as that produced with endogenously synthesized antigen.</p>		

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-1-

Enhancing the Association of Exogenous Peptides with Class I MHC Molecules on Immune System Cells.

#### Government Rights In Invention

5           This invention was made with the support of Government Grants AI 20248 and CA 46967 from the National Institutes of Health. The government of the United States of America has certain rights in this invention.

#### 10       Technical Field of Invention

          The present invention relates to a method for enhancing the association of exogenous peptides with class I MHC molecules on the surface of antigen presenting cells of the immune system and to the  
15       extension of this methodology to vaccination with synthetic peptides to prime for cytotoxic T lymphocyte response.

#### Background of the Invention

          Vaccines have long been utilized by man to aid  
20       in the prevention of infectious diseases in both humans and animals. Active immunization procedures have more recently been tested in other areas, such as the prevention and treatment of malignant disorders. The use of vaccines is based upon the  
25       stimulation of the specific immune response, which includes both humoral and cellular components. Although vaccination is often considered in terms of

its ability to stimulate antigen-specific antibody, vaccine effectiveness is not necessarily determined by the induction of serum antibody alone (humoral factors). Rather it is determined by the demonstration of enhanced protection against disease, which may include cytotoxic T lymphocyte response. Cytotoxic T lymphocytes provide an effective defense against many intracellular pathogens. In some cases, full protection can be achieved even in the absence of an antibody response.

Vaccines have historically been prepared by killing or attenuating pathogenic organisms, e.g. viruses, and then injecting the resulting virus particles into a patient or host mammal. One of the major problems with these vaccines is that they always involve an inherent threat that the virus may not be sufficiently attenuated or killed. There is thus the potential for the vaccine itself to cause the disease against which protection is sought. Another limitation of this approach is that most pathogens, when killed, do not effectively stimulate cytotoxic T lymphocyte immunity.

The threat of unattenuated pathogens can sometimes be overcome by using a specific component of the pathogen. The component used is typically a protein from, for example, a viral capsid or envelope which forms an outer portion of the virus.

As with the attenuated vaccines however, this approach is also limited in that most protein antigens which are introduced into the extracellular fluid do not effectively stimulate cytotoxic T lymphocyte immunity. In addition, this method also poses the possibility of a pathogenic response. A still further limitation is that the thus-produced vaccine may include antigens that compete with or are detrimental to the immune response and may cause undesirable side effects.

More recently, certain pathogen-related proteins have been immunologically mimicked with synthetic polypeptides whose amino acid sequence corresponds to that of an antigenic determinant domain of the pathogen-related protein. For example, U.S. Patent No. 4,974,168 describes leukemia associated immunogens that are relatively short peptide sequences corresponding to the antigenic determinant domains of a leukemia associated virus envelope protein. Similarly, other peptide immunogens have been reported by Sutcliffe et al, Nature, Vol. 287, pp. 801-805 (1980); Lerner et al, Proc. Natl. Acad. Sci. U.S.A., Vol. 78, pp. 347-47 (1981) and Bittle et al, Nature, Vol. 298, pp. 30-33 (1982). The peptide sequence of a natural protein can be determined either from the protein itself or from the nucleotide sequence of the genome encoding the protein.

Although peptide immunization can stimulate class II MHC-restricted T lymphocytes to help B lymphocytes secrete antibody (helper T cells) in a host, induction of a CTL response usually requires in vivo priming with infectious virus. Most studies have indicated that class I MHC cytotoxic T lymphocytes are rarely, if ever, induced with peptide immunizations. See, Bevan, "Stimulating Killer T Cells", Nature, Vol. 342, pp. 478-79 (November 1989). The general inability of synthetic polypeptides to prime cytotoxic T lymphocytes in vivo thus presents a formidable hurdle to the use of such peptides in effective vaccination.

There have been isolated reports in the literature of peptides which can prime CTL responses in vivo. For example, Deres et al recently reported that synthetic viral peptides covalently linked to tripalmitoyl-S-glycerylcysteinyl-seryl-serine can effectively prime influenza virus specific CTL in vivo. Nature, Vol. 342, pp. 561-62 (November 1989). Carbone and Bevan described in vivo priming of CTL with partial tryptic digests of ovalbumin (OVA) and a specific OVA peptide, OVA<sub>229-276</sub>, although all other OVA peptides tested, including peptides from a complete tryptic digest of OVA, were unable to prime CTL's. J. Exp. Med., Vol. 169, pp. 603-11 (March 1989). The most often provided explanation for the ability of a few peptides to serve as in vivo

immunogens for CTL is the ability of the peptides to enter into the cytoplasm of antigen-presenting cells, where they can enter the normal pathway that leads to association with class I molecules of the MHC.

By way of further background, cytotoxic T lymphocytes and T helper cells generally recognize processed antigenic peptides in association with class I and class II major histocompatibility (MHC) gene products, respectively, on the surface of antigen presenting cells (APC's). It is currently believed by those skilled in the art that the processed peptides arise from cleavage of protein antigens either in the cytosolic/endoplasmic reticulum or endosomal compartments of cells. Class I molecules are expressed on all cells and are meant to display a sample of internally synthesized peptides by the cells for surveillance by cytolytic T cells. It is generally believed that the cytosolic peptides which arise from antigens that are synthesized by cells bind to newly synthesized MHC molecules.

In contrast to the class I molecules, class II molecules have a limited expression in cells concerned with the presentation of foreign protein antigens to initiate the immune response. Antigens from the extracellular fluids are processed in



endosomes and subsequently bind to class II molecules. Antigens that are acquired from the extracellular fluid are thus presented in association with class II molecules, while those that are synthesized endogenously are presented in association with class I molecules. Germain, Nature, Vol. 332, pp. 687-89 (1986); Braciale et al., Immunol. Rev., pp. 95-114 (1987). A general inability of most antigens to enter into the class I antigen presentation pathway from extracellular fluid could thus account for the inability of such antigens to prime in vivo despite the achievement of effective priming of T helper cells, which are class II MHC-restricted. The reason for the general inability of synthetic peptides to prime class I MHC restricted T cells in vivo is not known.

As intimated above, the form in which antigen interacts with and is displayed by both class I and class II MHC is thought to be as a small peptide. If this is indeed the case, it would be a logical expectation that small peptides might bind to class I MHC molecules on the cell surface and thus bypass the intracellular processes and be presented to the immune system, when the antigen is presented in the form of small peptides in the extracellular fluid. This apparently does occur in vitro. For example, Maryanski et al demonstrated that certain peptides can sensitize class I MHC antigen presenting cells

for lytic action by antigen specific cytotoxic T lymphocytes at high doses of exogenous peptides in vitro. Nature, Vol, 324, No. 11 (1986). See also, Townsend et al, Cell, Vol. 44, pp. 959-68 (March 1986).

Peptide fragments at high concentrations can also prime naive populations of lymphocytes for cytotoxic T cell response in culture. Carbone et al, J. Exp. Med., Vol. 167, pp. 1767-79 (June 1988).

10 Experiments with fixed antigen presenting cells have shown that efficient access to the endogenous processing pathway of class I MHC antigen presenting cells is not required for antigen presentation to CTL in vitro under these culture conditions.

15 Although it is not clear why in vitro priming has been successful, there is some suggestion that the high concentrations of peptides typically employed in in vitro cultures may allow intracellular complexes to form or may drive the association of peptides directly with unoccupied class I molecules

20 on the cell surface. Use of high peptide concentrations in vitro may be sufficient for study of the events of target cell sensitization and priming for CTL response. Such culture conditions

25 may not, however, be the most efficient conditions with which to cause association of polypeptides and/or synthetic peptides with class I MHC molecules.

Despite the observations regarding in vitro sensitization and priming for CTL response, the events generally do not occur in vivo in a manner that has allowed stimulation of cytotoxic T lymphocyte response.

One possible explanation for this is that the concentration of circulating peptides is not maintained at a high enough level for this type of presentation to be effective in vivo. Even if sufficiently high concentrations of synthetic peptide could be maintained in a host organism to initiate CTL response, the cost of using such high concentrations of peptides with present technology would be prohibitive. Synthetic peptides are extremely expensive and vaccines requiring high concentrations of peptide would be unattractive for these reasons.

Accordingly, it is an object of the present invention to provide a method for enhancing the association of exogenous polypeptides with class I MHC-restricted molecules on the surface of antigen presenting cells.

Another object of the present invention is to provide a method for priming class I MHC-restricted cytotoxic T cell response with polypeptides.

Yet another object of the present invention is to provide a method for priming cytotoxic T cell response in vivo with polypeptides.

5 A still further object of the present invention is to provide an adjuvant that can be utilized with synthetic peptide vaccines to enhance the immunogenicity of pathogen-related peptides.

#### Summary of the Invention

10 These as well as other objects and advantages are accomplished by the present invention, which provides a method for enhancing the association of exogenously prepared polypeptides with class I MHC molecules on the surface of antigen presenting cells to thereby sensitize the target cells for class I  
15 MHC-restricted cytotoxic T cell response. The method of the present invention involves obtaining the polypeptides of interest and presenting the polypeptides to the appropriate antigen presenting cells in the presence of an elevated level of free  
20  $\beta_2$ -microglobulin. The concentration of  $\beta_2$ -microglobulin employed in the method of the present invention is elevated to a level higher than that normally present as free  $\beta_2$ -microglobulin in mammalian serum.

The method of the present invention can also be used to prime naive populations of T lymphocytes for antigen-specific class I MHC-restricted cytotoxic T cell response with exogenous polypeptide and is  
5 useful in both in vitro and in vivo applications. In accordance with the present invention, a new use for purified  $\beta_2$ -microglobulin as an adjuvant for synthetic polypeptide vaccines is also provided. In vivo immunization with the synthetic peptides and  
10 free  $\beta_2$ -microglobulin can elicit an antigen-specific response as strong as that produced with endogenously synthesized antigen.

#### Brief Description of the Drawings

15 Figure 1 is a graphical illustration which shows that exogenous peptides associate with class I and class II MHC on the surface of antigen presenting cells (APC's). A titration of antigen was added to cultures with T-T hybridomas and either  
20 live or fixed APC's or without APC's, as indicated in the legend. In Figure 1A, microcultures were prepared with RF33.70 T-T hybrids (anti-OVA + K<sup>b</sup>) and EL4 APC's (an H-2<sup>b</sup>, Class I<sup>+</sup>, Class II<sup>-</sup> lymphoblastoid cell line); in Figure 1B, with  
25 RF33.70 T-T hybrids and LB27.4 APC's (an H-2<sup>bxd</sup>, Class I<sup>+</sup>, Class II<sup>+</sup>, B lymphoblastoid cell line) and toOVA and in Figure 1C, with 8D051.15 T-T hybrids (anti-OVA + IA<sup>d</sup>) with LB27.4 APC's and either native

OVA or tOVA. T cell activation was measured using an assay for interleukin-2 production. IL-2 production is expressed as the incorporation of  $^3\text{H}$ -thymidine into DNA ( $\text{CPM} \times 10^{-3}$ ) by HT2 cells  
5 cultured in supernatant from the antigen-treated cultures.

Figure 2 is a graphical illustration of the kinetics of association of exogenous peptides with class I and class II molecules. APC's were  
10 preincubated with tOVA for 3 hours or 18 hours and were subsequently fixed and added to cultures with T-T hybridomas. In Figure 2A, microcultures were prepared with RF33.70, OVA +  $\text{K}^b$  specific T-T  
hybrids with EL4 APC's, in Figure 2B, with RF33.70  
15 T-T hybrids with LB27.4 APC's, in Figure 1C, with 8DO51.15 OVA +  $\text{IA}^d$  specific T-T hybrids with LB27.4 APC's and in Figure 1d, LB27.4 were fixed, incubated with  $20\mu\text{g/ml}$  tOVA for the indicated length of time  
and added to cultures with DO11.10, OVA +  $\text{IA}^d$   
20 specific T-T hybrids. T cell activation was measured as in Figure 1.

Figure 3 is a graphical illustration which shows that endogenously processed antigens rapidly  
associate with class I and class II molecules. OVA  
25 was introduced into the cytoplasm of LB27.4 APC's by osmotic lysis of pinosomes. The antigen-loaded APC's were incubated at  $37^\circ\text{C}$  for the indicated

times, fixed and added to cultures with T-T hybrids as follows: In Figure 3A, with RF33.70; in Figure 3B, with DO11.10. T cell activation was measured as in Figure 1 above.

5           Figure 4 is a graphical illustration which shows that the association of exogenous peptides with class I MHC is uniquely dependent on serum. LB27.4 APC's were incubated with or without tOVA or loaded with OVA by hypotonic lysis of pinosomes.  
10           The indicated concentration of normal mouse serum (NMS) or fetal calf serum (FCS) was present during the peptide pulse or in the incubation following the OVA loading. The APC's were subsequently fixed and added to cultures with T-T hybrids as follows: in  
15           Figure 4A, with RF33.70 T-T hybrids with tOVA pulsed APC's; in Figure 4B, with DO.11.10 T-T hybrids with tOVA pulsed APC's and in Figure 4C, with RF33.70 cells with OVA loaded APC's. T cell activation was measured as above.

20           Figure 5 is a graphical illustration which shows that exogenous peptides rapidly associate with class I molecules in the presence of exogenous, purified  $\beta_2$ -microglobulin. APC's were incubated in the presence or absence of the indicated  
25           concentrations of  $\beta_2$ -microglobulin. After 2-3 hours

incubation at 37°C, the APC's were fixed and added to cultures with the RF33.70, OVA + K<sup>b</sup> specific T-T hybrid. In Figure 5A, LB27.4 APC's were pulsed with toVA for 3 hours with or without human  $\beta_2$ -microglobulin; in Figure 5B, EL4 APC's were pulsed for 2 hours with toVA and human  $\beta_2$ -microglobulin and in Figure 5C, EL4 APC's were pulsed for 0.5-2 hours with toVA and human  $\beta_2$ -microglobulin.

Figure 6 is a graphical illustration demonstrating that exogenous  $\beta_2$ -microglobulin enhances peptide association if present during, but not before peptide pulsing. APC's were incubated with  $\beta_2$ -microglobulin either before or during the peptide pulse with toVA. Fixed APC's were cultured with RF33.70 T-T hybrids and the supernatant was measured for interleukin 2 content. T cell activation was measured as in the foregoing Figures.

Figure 7 graphically illustrates that exogenous peptide is associated with class I MHC molecules that have undergone  $\beta_2$ -microglobulin exchange. APC's were pulsed with toVA in media containing exogenous  $\beta_2$ -microglobulin of either murine or human origin. After antigen pulsing, the APC's were incubated with monoclonal antibodies specific for D<sup>b</sup> (anti-D<sup>b</sup>), class I MHC molecules with murine  $\beta_2$ -microglobulin (anti-mH-2), or human  $\beta_2$ -microglobulin (anti-huB2M) and were then fixed and added to



5 cultures with RF33.70, OVA + K<sup>b</sup> specific T-T hybridoma. Percent inhibition was calculated based upon the formula  $[1 - (\text{CPM of experimental group} / \text{CPM of control group})] \times 100$ . The experimental groups were anti-mH-2 and anti-huB2M, control group was anti-D<sup>b</sup>. The response of the control group was 100%.

10 Figure 8 is a graphical illustration demonstrating that effective priming of CTL in vivo can be effected by presenting APC's with peptides and  $\beta_2$ -microglobulin. Individual age and sex matched mice, one mouse per group were injected subcutaneously in each flank (two sites per animal) with 94  $\mu\text{g}$  of tOVA with or without 47 $\mu\text{g}$  of purified  $\beta_2$ -microglobulin (Sigma Chemical Co., St. Louis, Mo.) in phosphate buffered saline (47 $\mu\text{l}$  per injection site). After 7 days, the mice were sacrificed and the spleens harvested. Splenocytes (30 x 10<sup>6</sup>) were then stimulated with 15 x 10<sup>6</sup> 20,000 gamma irradiated EG7 cells (a transfected EL4 cell synthesizing OVA) in 10 mls of 10% FCS-containing RPMI 1640 at 37°C. After five days of incubation, the restimulated cells were tested as effectors in a standard <sup>51</sup>Cr release assay. Targets were, in Figure 8A, EL4 cells (no antigen), or EL4 cells precultured in 300 mg/ml tOVA for 18 hours at 37°C in media with 10% FCS, and in Figure 8B, EG7 cells (a transfected EL4 cell synthesizing OVA). Effector

to target ratios were 11, 33 and 100. Data are expressed as % specific release versus effector to target (E:T) ratio.

#### Detailed Description of the Invention

5           The major histocompatibility complex (MHC) is a chromosomal region consisting of a series of genes that code for the cell surface expression of strong transplantation antigens. These transplantation  
10           antigens are, in general, glycoproteins that are present on the surface of most nucleated cells. The MHC in mammals is also in the region where the histocompatibility-linked immune response genes are located; hence the chromosomal segment not only  
15           controls the synthesis of transplantation antigens and graft rejection, but also influences immune responses to infectious challenge and susceptibility to the development of immunologically mediated diseases. The two MHC systems that have been most  
20           extensively characterized are the H-2 system in the mouse and the HLA system in humans.

          The MHC's of the mammals studied to date are remarkably similar, suggesting an evolutionary pressure for conservation of these genetic regions. In each instance, the genes code for two general  
25           types of antigen; class I and class II. The present

invention is generally related to the association of antigen with class I molecules.

5       The class I genes (HLA-A, B and C in humans, H-2K, D, L in mice) code for multi-determinant antigens which appear on the surface of cells as two peptide chains. Only the heavy chain is coded for by the MHC, and contains hypervariable regions analogous to the immunoglobulins. The heavy chain peptide consists of a large transmembrane  
10       glycoprotein of about 44K molecular weight (350 amino acids). This heavy chain is usually non-covalently associated with the light chain,  $\beta_2$ -microglobulin, a 100 amino acid, 12K molecular weight protein.  $\beta_2$ -microglobulin is encoded by  
15       genes on a separate chromosome than those coding for the class I heavy chains.

      It has been heretofore shown that exchange of  $\beta_2$ -microglobulin chains will occur in the presence of free  $\beta_2$ -microglobulin. Hyafil and Strominger,  
20       for example, demonstrated that radio-labeled urinary  $\beta_2$ -microglobulin is incorporated into the human histocompatibility complex HLA upon incubation of the  $\beta_2$  with papain-solubilized HLA. The radio-labeled urinary  $\beta_2$ -microglobulin was incorporated  
25       into HLA, where it was found to have substituted for the pre-existing  $\beta_2$  that had disassociated from the complex. Proc. Natl. Acad. Sci., U.S.A., Vol. 76,

No. 11 pp. 5834-38 (1979). Bernabeu et al demonstrated that  $\beta_2$ -microglobulin from fetal calf serum (FCS) or human serum used in cell culture can exchange  $\beta_2$ -microglobulin associated with HLA antigens and with mouse  $\beta_2$ -microglobulin on the cell surface. Nature, Vol. 308, pp. 642-45 (April 1984). The present invention is based upon the discovery that the binding of exogenous polypeptides to class I MHC molecules on the surface of antigen presenting cells occurs upon disassociation and reassociation of the  $\beta_2$ -microglobulin light chains, which has not heretofore been recognized.

The mechanism of the association of exogenous polypeptides is elucidated in the examples herein, and specifically, in Examples 1-4. These examples demonstrate that class I MHC molecules can associate exogenous polypeptides on the cell surface and can also associate antigen that has been processed intracellularly. The examples also show that, although the class I MHC molecules will associate exogenous polypeptides on the surface of antigen presenting cells, association of peptides with class I MHC is much less efficient than the association of endogenously processed antigen with class I MHC molecules. Elevated levels of free, exogenous  $\beta_2$ -microglobulin have been found, in accordance with

the present invention, to be necessary for the efficient association of peptides with class I MHC molecules on the surface of antigen-presenting cells. Although free  $\beta_2$ -microglobulin is inherently present in some serum-containing culture media, it has never been recognized or suggested that a component in the media might facilitate association of peptides with class I molecules. A role for free  $\beta_2$ -microglobulin has heretofore been unknown.

10           The present invention thus provides a method for markedly enhancing the association of exogenous polypeptides to class I MHC molecules on the surface of antigen presenting cells. The method comprises the steps of obtaining polypeptides of interest and  
15           presenting the polypeptides extracellularly to the appropriate antigen presenting cells in the presence of elevated levels of exogenous, free  $\beta_2$ -microglobulin. This method can be utilized to enhance the efficiency of the sensitization of  
20           target cells with antigen in vitro, and can also be utilized to prime naive populations of T lymphocytes for antigen-specific cytotoxic T cell response, both in vitro and in vivo. As used herein, the term "sensitize" refers to the association of  
25           polypeptides with MHC molecules on antigen presenting cells. The sensitization of class I MHC target or antigen presenting cells is generally determined by assaying for cytotoxic T lymphocyte

response using antigen specific CTL's, which recognize antigen in association with the class I restriction pattern. "Priming" refers to the initiation or activation of cytotoxic T lymphocyte response from a naive population of lymphocytes, i.e. a population of T lymphocytes that has not previously been exposed to the antigen. The method of the present invention is useful in the study of class I MHC antigen presentation and cell cell interaction and is also useful for enhancing the immunogenicity of synthetic peptides in vaccines.

The polypeptides that can be used in accordance with the method of the present invention are not particularly limited. As used herein, the words "polypeptide" and "peptide" are used interchangeably and designate a linear sequence of amino acids connected one to the other by peptide bonds between the alpha amino and carboxy groups of adjacent amino acids. The polypeptides can be of a variety of lengths, either in their neutral (uncharged) form or in forms such as their salts and either free of modifications such as glycosylations, side chain oxidation or phosphorylation or containing such modifications. Also included in the definition are polypeptides modified by additional substituents attached to the amino side chains, such as glycosyl units, lipids or inorganic ions such as phosphates as well as chemical modifications of the chains.

Thus, the term "polypeptide" or its equivalent is intended to include the appropriate amino acid sequence referenced, subject to the foregoing modifications, which do not destroy its  
5 functionality.

In general, the polypeptides used in accordance with the method of the present invention will be useful in research in the area of class I MHC restricted lymphocyte biology, in the development of  
10 synthetic class I immunogenic polypeptides that mimic naturally processed antigen in vitro and/or in vivo, and in vaccination with class I synthetic polypeptides designed to elicit a cytotoxic T lymphocyte response. For in vitro applications, the  
15 polypeptides can be virtually any exogenous, e.g. extracellularly produced, polypeptide that is of interest. Determination of an appropriate concentration of peptide for in vitro applications is conventional and well within the skill of the  
20 art.

For the in vivo applications, the polypeptides will preferably encompass epitopes that can be recognized by class I MHC restricted T lymphocytes. The peptides can be used in the immunization alone,  
25 or bound to a carrier or other haptenic molecule. Such polypeptides are sometimes hereinafter referred to as class I polypeptides. As used herein, the

term "epitope" refers to that portion of a molecule, usually a protein, that is specifically bound by a T cell receptor or an antibody combining site. The term is also used interchangeably with "antigenic determinant". In most instances, the polypeptide used in accordance with the present invention will be one which has been shown to prime cytotoxic T lymphocyte response in vitro. For example, various synthetic peptides derived from the influenza virus have been shown to induce cytolytic T cell activity in vitro, but not in vivo. These include peptides from the influenza nucleoprotein composed of residues 365-80 (NP365-80), NP50-63, and NP147-58 and peptides from influenza hemagglutinin HA202-21 and HA523-45, defined previously in class I restricted cytotoxicity assays. See, Perkins et al, J. Exp. Med., Vol. 170, 279-289 (July 1989). Enhanced efficiency of association of such polypeptides to class I molecules on antigen presenting cells in vivo has major implications for the use of these synthetic peptides as influenza vaccines. Other examples of synthetic peptides containing known epitopes that can be recognized by CTLs include influenza strain A/Jap/57 hemagglutinin protein, residues 508-530; influenza strain A/PR8/34 nucleoprotein residues 360-385; and HIV Pol (reverse transcriptase) residues 203-219. This list of peptides is exemplary only and is not intended to



limit in any way the class I peptides with which the method of the present invention can be employed. Rather, in accordance with the present invention, the association of the class I peptides to class I  
5 molecules on the surface of antigen presenting cells can be enhanced, to thereby enhance the immunogenicity of any class I polypeptide immunogen.

The polypeptides used in accordance with the method of the present invention can be prepared in  
10 any one of a number of conventional ways. Because they will generally be short sequences, they can be prepared by chemical synthesis using standard techniques. Particularly convenient are the solid phase techniques. Automated peptide synthesizers  
15 are commercially available, as are the reagents required for their use. Alternatively, the peptides can be prepared, as in the Examples herein, by enzymatic digestion or cleavage of naturally occurring proteins. The peptides can also be  
20 prepared using recombinant techniques known to those of skill in the art.

The source of the  $\beta_2$ -microglobulin employed in accordance with the method of the present invention is not critical. The  $\beta_2$ -microglobulin can thus be  
25 obtained, for example, from human, murine, bovine, equine or other mammalian serum or body fluids

normally containing  $\beta_2$ -microglobulin. Purified human  $\beta_2$ -microglobulin is available commercially, for example from Sigma Chemical Co., St. Louis, MO., and can be readily obtained. Alternatively,  $\beta_2$ -microglobulin can be isolated and purified from serum or other body fluids using conventional techniques or can be produced by recombinant techniques based upon the introduction of  $\beta_2$ -microglobulin genes into appropriate expression systems. The gene-encoding  $\beta_2$ -microglobulin has previously been cloned. In addition, its sequence is known, thus allowing for the isolation of a DNA clone by persons skilled in the art.

When used as an adjuvant for vaccination with polypeptides, it is preferable, although not necessary, to use  $\beta_2$ -microglobulin originating from the same species as the vaccine recipient in order to reduce the possibility of an immune response to the exogenously produced free  $\beta_2$ -microglobulin.

In accordance with the present invention, an increased number of antigen-class I complexes will occur in the presence of  $\beta_2$ -microglobulin at any point in time. It is expected that more complexes will form at higher concentrations of  $\beta_2$ -microglobulin. More antigen-MHC-class I complexes will result in a stronger and more persistent

stimulation, and hence, higher concentrations of  $\beta_2$ -microglobulin are most desirable.

For in vitro applications, the concentration of  $\beta_2$ -microglobulin should be raised to a level higher than that inherently present in serum-containing culture media. For example, cell culture media typically include 10% fetal calf serum, in a 1:10 dilution with another medium, for example RPMI-1640. (Since  $\beta_2$ -microglobulin is present in fetal serum at a level of about 10-fold higher than that present in adult serum, a 10% fetal serum-containing media roughly approximates conditions in vivo in adult mammalian serum). While the culture media thus contains some free  $\beta_2$ -microglobulin, it has been found in accordance with the present invention, that such concentration is insufficient to cause the efficient association of exogenous peptides with class I MHC molecules. The concentration of free  $\beta_2$ -microglobulin should be increased by at least 2 to 10 fold that normally present in the culture media to achieve the most efficient association in terms of the number of antigen-MHC complexes.

The concentration of free  $\beta_2$ -microglobulin in adult mammalian serum is very low and hence reassociation of free  $\beta_2$ -microglobulin with class I MHC molecules is limited. For in vivo applications, the amount of free  $\beta_2$ -microglobulin must be

sufficient to increase the concentration of free  $\beta_2$ -microglobulin in the serum above the level normally present in mammalian serum in order to favor the binding of  $\beta_2$ -microglobulin, and thereby peptides, to class I MHC heavy chains and stimulate an immune response. The concentration of  $\beta_2$ -microglobulin can be elevated systemically, i.e. throughout the body by increased levels in the blood, or locally at the site of injection. The latter is more attractive clinically and economically, and hence is preferred. While the required amount of  $\beta_2$ -microglobulin may vary to some degree from species to species, normal concentration ranges are readily ascertainable and in many instances are available in the published literature. Adult human  $\beta_2$ -microglobulin, for example, is normally present in serum an amount of about 1.5-2  $\mu\text{g/ml}$ . See, "Trace Components of Plasma: Isolation and Clinical Significance," Jamieson, G.A. Ed., Alan R. Liss, Inc. Publisher, New York, N.Y. (1976). The phrase "elevated  $\beta_2$ -microglobulin concentration" thus refers to a concentration that is greater than that normally present in mammalian serum and which is sufficient to stimulate an immune response. Precise amounts of  $\beta_2$ -microglobulin can be tailored to the particular application and peptides used in the immunization.

At high local concentrations of  $\beta_2$ -microglobulin, such as that employed in Example 5 herein, no adverse affects are observed.

5 In one embodiment of the present invention, the efficiency of the association of exogenous peptides with class I molecules on the surface of antigen presenting cells in vitro can be enhanced by presenting the peptides of interest to the antigen presenting cells in the presence of an elevated  
10 concentration of  $\beta_2$ -microglobulin. In the case of sensitization of antigen presenting cells for presentation to antigen-specific cytotoxic T lymphocytes, the antigen presenting cells share class I molecules with the host strain from which  
15 the antigen-specific CTL's were developed. The ratio of cytotoxic T lymphocytes (or CTL T-T hybridomas) to antigen presenting cells in the culture is not particularly limited. Generally, there are about 1-100 times the number of CTLs to  
20 antigen presenting cells, with a preferred ratio of about 100:1 to about 10:1 for normal CTL populations and 10:1 to  $\geq 1:1$  for clonal CTL populations. Peptides are presented to the antigen presenting cells in the presence of an elevated level of  $\beta_2$ -  
25 microglobulin. Preincubation of APC's with the  $\beta_2$ -microglobulin prior to incubation with the peptides is not sufficient. Both the exogenous peptides and

the free  $\beta_2$ -microglobulin must be in the presence of the APC's for enhanced association.

Similarly, in the priming of naive cells with antigen presenting cells in association with peptides, the antigen presenting cells share class I molecules with the strain from which the naive cells were obtained. Naive cells can be, for example, spleen cells from unprimed mice. Other sources of naive T lymphocyte populations include peripheral blood lymphocytes or lymph node cells.

Antigen presenting cells for in vitro applications include macrophages, B lymphocytes, T lymphocytes or the like bearing the appropriate class I MHC molecule. In accordance with this embodiment, the association of peptides with class I molecules on the surface of antigen presenting cells can be enhanced for virtually any exogenous peptide/MHC class I combination.

In another embodiment,  $\beta_2$ -microglobulin may serve as an adjuvant in vaccination of a host organism with exogenous peptides which mimic epitopes of a pathogen against which protection is sought. In accordance with this embodiment, the host organism is immunized with the synthetic peptides and free  $\beta_2$ -microglobulin such that the

antigen presenting cells of the host organism are presented with the peptides and  $\beta_2$ -microglobulin. When administered in effective amounts in the presence of elevated levels of free  $\beta_2$ -

5 microglobulin, the peptides are capable of initiating effector and memory class I restricted cytotoxic T lymphocytes which immunoreact with the polypeptides or with pathogen related immunogen that corresponds in amino acid sequence to the exogenous

10 polypeptides used in the vaccine on the surface of antigen presenting cells. In accordance with the method of the present invention, it is possible to achieve more peptide/class I MHC complexes and therefore stronger stimulation of CTL response than

15 with other known methods.

The effective amount of exogenous polypeptide in a unit dose depends upon a number of factors. Included among these factors are the nature of the carrier, if one is used, the amount of  $\beta_2$ -

20 microglobulin, the number of inoculations to be used and, to some extent, the body weight of the mammal to be immunized. Individual local inoculations will typically contain about 50 micrograms to about 5 milligrams of polypeptide exclusive of any carrier

25 to which the peptides may be linked. For systemic administration, inoculations may typically contain about 0.5 to 3 mg/kg of body weight. To achieve a useful level of protection in a population with

synthetic peptides, a variety of peptide epitopes, derived from various subunits of the pathogen preferably will preferably be employed, since MHC molecules are known to be polymorphic.

5 Alternatively, where a specific peptide is known to be reactive with a particular MHC class I molecule, individuals with that MHC-specificity can be specifically immunized with the peptide. In addition to the class I restricted peptides, the  
10 vaccine can additionally include class II MHC-restricted peptides, the latter to supply help for the CTL response.

The preparation of vaccines which contain peptide sequences as active ingredients is well  
15 understood in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions. The active ingredients are often mixed with excipients which are pharmacologically acceptable and compatible with the active  
20 ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof, with saline being particularly preferred. In addition, and in accordance with the present invention, the vaccine  
25 contains free  $\beta_2$ -microglobulin as an adjuvant which enhances the effectiveness of the vaccine.



The vaccines are conventionally administered by injection, for example either subcutaneously or intramuscularly. In accordance with the present invention, the exogenous peptides which are the active ingredients of the vaccine are presented to antigen presenting cells of the immune system in the presence of elevated levels of free  $\beta_2$ -microglobulin. Thus, in one embodiment, the exogenous peptides and free  $\beta_2$  can be prepared as an injectable and simultaneously injected into the mammal to be immunized. The peptides and  $\beta_2$  can either be free in the liquid solutions or suspensions or can be encapsulated in a depot or other controlled release vehicle. Alternatively, the exogenous peptides and free  $\beta_2$  can be injected sequentially. Booster injections can be given, if needed. The critical feature is that the peptides are presented to the APC's in the presence of free  $\beta_2$ -microglobulin and any method utilized to accomplish such result is considered to be within the spirit and scope of this invention.

The present invention will be more clearly understood from the following specific examples.

**Example 1****Association of Exogenous Peptides  
With Class I MHC Molecules**

5        Although it has been generally assumed that  
exogenous peptides associate directly with class I  
MHC molecules at the cell surface, this assumption  
has not been formally tested in most systems,  
because conventional cytolytic assays require intact  
antigen presenting cells. There is recent evidence,  
10       however, that exogenously added peptides may  
associate intracellularly with newly synthesized  
class I molecules. This example demonstrates that  
peptides can associate with class I MHC molecules at  
the APC cell surface.

15       Microcultures were prepared with  $5-10 \times 10^4$  T-T  
hybridomas with a titration of tOVA and with or  
without  $5 \times 10^4$  live or fixed antigen presenting  
cells in RPMI 1640-10% fetal calf serum (FCS) media.  
The T-T hybridomas were RF33.70, an anti-OVA +  $K^b$   
20       hybrid which secretes interleukin-2 upon appropriate  
antigenic stimulation, and 8D051.15, an anti-OVA +  
 $IA^d$  hybrid. Antigen presenting cells were EL4  
cells, (an  $H-2^b$ , Class I+, Class II-, T  
lymphoblastoid cell line) or LB27.4 cells, an  $H-$   
25        $2^{bx_d}$ , Class I+, Class II+, lymphoblastoid cell line.  
The antigen presenting cells were chemically fixed  
with glutaraldehyde as previously described.  
Shimonkevitz et al., J. Exp. Med., Vol. 158, pp.  
303-16 (1983).

A titration of exogenous peptides, tOVA, as set forth in the legend to Figures 1 A-C herein, was added to the cultures and the cultures were incubated for 18 hours at 37°C. After 18 hours, a  
5 100µl aliquot of supernatant was removed and assayed for interleukin-2 (IL-2) content with HT2 cells as previously described. Rock et al., J. Exp. Med., Vol. 157, pp. 1618-34 (1983). As illustrated in  
10 Figure 1, exogenous peptides associate with class I and class II molecules on the surface of antigen presenting cells.

As also illustrated in Figure 1, fixed APC's present tOVA to RF33.70 (Figures 1A and B). The chemical fixation in this example is adequate  
15 because the treated APC's are unable to process native antigen for presentation with class II (Fig. 1C) or class I (Fig. 3) MHC molecules and were otherwise metabolically inactive. These results demonstrate that peptides can associate with K<sup>b</sup>  
20 molecules on the antigen presenting cell surface.

### Example 2

#### Kinetics of Peptide-MHC Association

This example examines the kinetics of formation of peptide-MHC complexes with class I versus class  
25 II molecules.

Live or fixed antigen presenting cells were preincubated with tOVA for 3 or 18 hours at the concentrations indicated in Figure 2 and were subsequently fixed and added to cultures with T-T hybridomas as follows.

- 5       A.    RF33.70, OVA + K<sup>b</sup> T-T hybridoma with EL4 APC's
- B.    RF33.70 T-T hybrids with LB27.4 APC's
- C.    8DO51.15, OVA + IA<sup>d</sup> specific T-T hybrids
- 10        with LB27.4 APC's and
- D.    LB27.4 APC's were fixed, incubated with
- 20µg/ml t OVA and added to cultures with
- DO.11.10, OVA + IA<sup>d</sup> specific T cells.

15       Live or fixed APC's were incubated with the indicated concentration of tOVA in RPMI 1640 culture media with 10% fetal calf serum. After 3 or 18 hours, the APC's were washed and the live APC's were fixed. Microcultures were prepared with  $2-5 \times 10^5$  (A-C) or  $5 \times 10^4$  (D) fixed APC's and  $5 \times 10^5$  T-T

20       hybrids. Antigen presenting cells were fixed with paraformaldehyde as previously described. Faló, et al., Proc. Natl. Acad. Sci., U.S.A., Vol. 82, pp. 6647-51 (1985). After 20 hours of incubation at

25       37°C, a 100 µl aliquot of supernatant was removed and assayed for IL-2 content.

A marked difference in the behavior of class I versus class II molecules was observed. The association of tOVA with Ia<sup>d</sup> molecules (class II) on LB27.4 APC's occurs rapidly (Fig. 2C) even at low peptide concentrations and when the APC's are fixed (Fig. 2D) which indicates that there are Ia molecules on the cell surface that are highly receptive to exogenous peptides. In contrast, the association of tOVA with class I MHC molecules on the same APC's requires extended incubation in relatively high concentrations of peptide (Fig. 2B). The slow association between exogenous OVA peptide and K<sup>b</sup> molecules appears to be a general phenomenon, as it is observed with both B (Fig. 2B) and T (Fig. 2A) APC's. The EL4 APC's are more active than LB27.4, which may reflect the higher level of expression of K<sup>b</sup> on the former. This example thus shows that exogenous peptides associate much more readily with class II molecules than class I molecules on the surface of APC's.

### Example 3

#### Endogenous Peptide Class I MHC Association

This example demonstrates the kinetics of formation of peptide-MHC complexes arising from endogenously produced antigen. OVA was introduced into the cytoplasm of LB27.4 APC's by osmotic lysis of pinosomes, as previously described. See, Moore, et al., Cell, Vol. 54, pp. 777-85 (1988); Okada, et

al., Cell, Vol. 29, pp. 33-41 (1982). To summarize,  
LB27.4 APC's were incubated with 30mg/ml OVA in  
hypertonic buffer for 10 minutes at 37°C, followed  
by a 3 minute incubation in hypertonic buffer and  
5 were then washed at 4°C. The OVA loaded APC's were  
incubated at 37°C for 0-60 minutes and were then  
fixed with paraformaldehyde. Microcultures were  
prepared with  $10 \times 10^4$  or  $1.25 \times 10^4$  APC's and  $5-10$   
 $\times 10^4$  T-T hybridomas. T-T hybrids were RF33.70 and  
10 DO.11.10, respectively. After 20 hours incubation,  
100  $\mu$ l aliquots of supernatant were collected and  
assayed for IL-2 content as previously described.

The example shows the appearance of OVA-K<sup>b</sup>  
complexes on the APC surface is detectable after 15-  
15 30 minutes and is near maximal by 1 hour following  
antigen loading (Fig 3A). The rapid appearance of  
antigen-class I MHC complexes is observed even under  
limiting conditions of antigen. It should be noted  
that the ability of the OVA-loaded APC's to present  
20 antigen required incubation at 37°C and was  
inhibited by fixation, which presumably reflects a  
requirement for intracellular processing or  
transport. These results indicate that peptide-  
class I MHC complexes can form rapidly, which  
25 resembles the behavior of class II molecules (Fig.  
3B) more closely than that of the mature class I MHC  
molecules on the APC surface.

**Example 4****B<sub>2</sub>- microglobulin and Peptide Association**

a. The association of exogenous peptide with class I molecules is uniquely dependent on serum.

5 LB27.4 APC's were either incubated with or without toVA or loaded with OVA by the hypotonic lysis of pinosomes. The APC's in this example were adapted to grow in serum free media (SFM) (OPTIMEM media, GIBCO, Grand Island, N.Y.) supplemented with 1% SP-nutridoma (Boehringer Mannheim, Indianapolis, IN)

10 supplemented with 1% normal mouse serum (NMS). APC's were incubated for 18 hours at 37°C, with (a) 400µg/ml or (b) 100 µg/ml toVA in SFM supplemented with 1 or 10% normal mouse serum (NMS) or fetal calf

15 serum or were loaded with OVA (20mg/ml) via osmotic lysis of pinosomes and were incubated for 1 hour at 37°C in SFM supplemented with 1% NMS or 10% FCS. The APC's were subsequently fixed with paraformaldehyde. Microcultures were prepared with 5-10 x 10<sup>4</sup>

20 T-T hybridomas and the number a APC's, as indicated in Figure 4. The microcultures were handled as previously described and assayed for IL-2 content.

The results show that the formation of peptide-class I MHC complexes, that occurs upon extended

25 incubation of APC's with exogenous peptide, is markedly influenced by the exogenous serum concentration. As shown in Fig. 4A, toVA associates with class I in the presence of 10%, but not in 1% serum. The critical variable is the concentration

of serum, rather than the species of origin of the serum, although the heterologous fetal serum may be more active. Moreover, when the same APC's are loaded with native OVA, the generation of OVA-class I MHC complexes from endogenously processed antigen and intracellular class I molecules is not affected by the exogenous serum (Fig. 4C).

In contrast to class I, the association of peptides with class II molecules on the surface of APC's is not dependent on the exogenous serum (Fig 4B). These results indicated that a component in serum may be necessary or facilitate peptide association uniquely with class I MHC molecules on the APC surface.

b. The effect of exogenous  $\beta_2$ -microglobulin. This example demonstrates that the efficiency of association of exogenous peptide with  $K^b$  class I MHC can be significantly enhanced by increasing the amount of free  $\beta_2$ -microglobulin present in the incubation media. EL4 or LB27.4 APC's were pulsed with tOVA in media containing low amounts of serum and in the presence or absence of purified human  $\beta_2$ -microglobulin as follows.

APC's were incubated with or without 100 or 300  $\mu$ g/ml tOVA in complete SFM, 1% NMS, as described above, and with the addition of purified  $\beta_2$ -



microglobulin (Sigma Chemical Co., St. Louis, MO.)  
at (a) 2.5  $\mu\text{g/ml}$ , (b) 0.1-9  $\mu\text{g/ml}$  and (c) 9  $\mu\text{g/ml}$ .  
After 2-3 hours of incubation at 37°C, the APC's  
5 were fixed with paraformaldehyde and added to  
cultures with the RF33.70, OVA- $\text{K}^b$ . specific T-T  
hybridomas. Microcultures were prepared with 5-10 x  
10<sup>4</sup> APC's and handled as previously described.

The results of this study demonstrate that  
peptide rapidly associates with class I MHC  
10 molecules in the presence of exogenous, purified  $\beta_2$ -  
microglobulin. In the presence of exogenous  $\beta_2$ -  
microglobulin, there is extremely effective pulsing  
of class I MHC molecules on both APC's (Fig. 5).  
Under these conditions, the association of peptide  
15 is rapid and can be detected after 30-60 minutes  
(Fig. 5C). The optimal concentration of  $\beta_2$ -  
microglobulin under these conditions is greater than  
9  $\mu\text{l/ml}$ . At low concentrations, such as are present  
in typical culture media containing 10% serum, the  
20 formation of OVA-class I molecules is not detectable  
in this time period (Fig. 5B). The species of  $\beta_2$ -  
microglobulin is not critical, as evidenced, for  
example, by Figures 4A and 5. Thus, in accordance  
with the present invention, the association of  
25 peptide with class I MHC molecules on the surface of  
antigen presenting cells can be enhanced by  
increasing the amount of free  $\beta_2$ -microglobulin to a  
concentration greater than that normally found in

culture media. Since fetal serum contains up to 10 times more  $\beta_2$ -microglobulin than adult serum, the amount of  $\beta_2$  in media supplemented with 10% serum approximates the conditions in vivo.

5           c. Preincubation of APC's with  $\beta_2$ -  
microglobulin. This example demonstrates that the  
association of exogenous peptides with class I  
molecules requires that the exogenous  $\beta_2$ -  
10 microglobulin be present at the time of peptide  
pulsing. APC's were incubated with  $\beta_2$ -microglobulin  
either before or during pulsing with the exogenous  
peptide, tOVA. EL4 APC's were incubated with or  
without 2.5  $\mu$ g/ml of purified human  $\beta_2$ -microglobulin  
15 for 18 hours at 37°C and then washed. The APC's  
were subsequently incubated with or without 200  
 $\mu$ g/ml tOVA in SFM, 1% NMS, in the presence or  
absence of 2.5  $\mu$ g/ml human  $\beta_2$ . After 3 hours  
incubation at 37°C, the APC's were fixed with  
paraformaldehyde. Micro-cultures were prepared as  
20 described in Example 4a above.

As illustrated in Figure 6, APC's that have  
been preincubated with purified  $\beta_2$ -microglobulin,  
washed and then incubated with peptide for three  
hours do not present tOVA in association with class  
25 I molecules. The association of tOVA with K<sup>b</sup>  
requires that the exogenous  $\beta_2$ -microglobulin be  
present at the time of peptide pulsing.

The results of this study demonstrate that exogenous  $\beta_2$ -microglobulin enhances peptide association if present during, but not before, peptide pulsing with exogenous peptide.

5           d. Exogenous peptide is associated with class I MHC molecules that have undergone  $\beta_2$ -microglobulin exchange. This example indicates that binding of exogenous peptide occurs during the exchange of MHC  $\beta_2$ -microglobulin with exogenous  $\beta_2$ -microglobulin.  
10       APC's were pulsed with tOVA in media containing exogenous  $\beta_2$ -microglobulin of human or murine origin. After antigen pulsing, the APC's were incubated with monoclonal antibodies (MAb's) specific for D<sup>b</sup> (Anti-D<sup>b</sup>), class I MHC molecules  
15       with murine  $\beta_2$ -microglobulin (anti-mH-2) or human  $\beta_2$ -microglobulin (anti-huB2-M), and were then fixed and added to cultures with the RF33.70, OVA + K<sup>b</sup> specific T-T hybridoma.

Specifically, LB27.4 APC's were incubated with  
20       400 $\mu$ g/ml tOVA in SFM supplemented with either 10% NMS (as a source of murine  $\beta_2$ -microglobulin) or 1% NMS and 2.5  $\mu$ g/ml human  $\beta_2$ -microglobulin for 18 hours at 37°C. The antigen pulsed APC's were washed, incubated in a saturating amount of 28.14.8S  
25       (anti-D<sup>b</sup>), M1/42 (anti-murine class I heterodimers) or BBM.1 (anti-human  $\beta_2$ -microglobulin), for 30 minutes at 4°C, and then were fixed. Microcultures

were prepared with  $1 \times 10^5$  NMS cultured APC's or  $3 \times 10^6$  human  $\beta_2$  cultured APC's and  $10^5$  T-T hybrids and were handled as described in Example 1. No additional MAb was added to these cultures. The APC's pulse more strongly in the presence of human  $\beta_2$ -microglobulin and therefore the number of APC's added to the cultures was adjusted to give approximately equivalent responses. Under these conditions, the response of the RF33.70 hybrid to APC's pulsed with NMS and human  $\beta_2$ -microglobulin was  $35 \times 10^3$  counts per minute (CPM) and  $41 \times 10^3$  (CPM), respectively, in the control (28.14.8S) groups. The response of the APC's not exposed to antigen was  $0.4 \times 10^3$  CPM.

The results show that T cell responses to APC's pulsed with tOVA and mouse  $\beta_2$ -microglobulin are inhibited by the M1/42 MAb, that is specific for murine-murine, class I heterodimers, and are unaffected by the BBM.1 MAb, that is specific for human  $\beta_2$ -microglobulin (Fig. 7). In contrast, a reciprocal pattern of inhibition was observed when the same APC's were pulsed with tOVA in the presence of exogenous human  $\beta_2$ -microglobulin (Fig. 7). The inhibition that was observed with the anti-human  $\beta_2$ -microglobulin MAb was only partial. However, this does not reflect the presence of peptide bound to murine-murine class I heterodimers, since the M1/42 MAb does not cause inhibition. The partial effect

of the BBM.1 MAb could reflect the location of the bound MAb (distant from the peptide/T cell receptor interaction sites), elution of the bound MAb and/or exchange with bovine  $\beta_2$ -microglobulin. Taken  
5 together, these results indicate that the association of exogenous peptides with class I molecules on the cell surface requires the disruption and reformation of the class I MHC heterodimer.

10

#### Example 5

##### In Vivo Immunization

This experiment establishes that the method of the present invention can be utilized to induce cytotoxic T lymphocyte response in vivo.

15

Individual age and sex matched C57BL6 mice, one per group, were injected subcutaneously in each flank (two sites per animal) with 94  $\mu$ g of tOVA with or without 47 $\mu$ g of purified  $\beta_2$ -microglobulin (Sigma Chemical Co., St. Louis, Mo.) in phosphate buffered  
20 saline (47 $\mu$ l per injection site). The total amount of tOVA injected into each animal was thus 188  $\mu$ g; total  $\beta_2$ -microglobulin was 94  $\mu$ g. After 7 days, the mice were sacrificed and the spleens harvested. Splenocytes ( $30 \times 10^6$ ) were then stimulated with 15  
25  $\times 10^6$  20,000 gamma irradiated EG7 cells (a transfected EL4 cell synthesizing OVA) in 10 mls of 10% FCS-containing RPMI 1640 at 37°C.

After five days of incubation, the restimulated cells were tested as effectors in a standard <sup>51</sup>Cr release assay. Targets were EL4 cells (no antigen), EL4 cells precultured in 300 mg/ml tOVA for 18 hours at 37°C in media with 10% FCS, or EG7 cells (a transfected EL4 cell synthesizing OVA).

As illustrated in Figures 8A and B, injection of tOVA and  $\beta_2$ -microglobulin into unprimed mice induced a good cytotoxic T lymphocyte response; restimulated spleen cells lysed EL4 APC's in the presence of tOVA, but not in the presence of APC's alone. Consistent with previous reports in the literature (See, Carbone and Bevan, J. Exp. Med., Vol. 169, pp. 603-11 (March 1989)), no priming was obtained with tOVA in the absence of  $\beta_2$ -microglobulin. This control establishes the criticality of an elevated level of  $\beta_2$ -microglobulin for in vivo priming with peptides. Figure 8B illustrates that priming with peptides and  $\beta_2$ -microglobulin stimulated CTL that can recognize the naturally produced/ processed form of OVA (EG7 cells), which is important for vaccine applications.

The results of this study demonstrate that normal APC's exposed in vivo to exogenous peptide in the presence of an elevated level of  $\beta_2$ -microglobulin will acquire the peptides in a manner

which allows for the priming of antigen specific cytotoxic T lymphocytes.

5           From the above description, it is apparent that the objects of the present invention have been achieved. While only certain illustrative embodiments have been set forth, alternative embodiments will be apparent from the above description to those skilled in the art.

What is claimed is:

1. A method for enhancing the association of exogenous peptide with class I MHC molecules on the surface of antigen presenting cells comprising the steps of:

- 5           (a) obtaining the exogenous peptides and  
          (b) presenting the exogenous peptides to the antigen presenting cells in the presence of an elevated amount of free  $\beta_2$ -microglobulin sufficient to stimulate an immune response.

2. A method according to claim 1 wherein the  $\beta_2$ -microglobulin is selected from the group consisting of purified human  $\beta_2$ -microglobulin, purified murine  $\beta_2$ -microglobulin, purified bovine  $\beta_2$ -microglobulin and purified equine  $\beta_2$ -microglobulin and mixtures thereof.
- 5

3. A method according to claim 2, wherein the  $\beta_2$ -microglobulin is purified human  $\beta_2$ -microglobulin.

4. A method according to claim 1, wherein the exogenous peptides comprise complete trypsin-digested ovalbumin peptides.

5. A method according to claim 1, wherein the exogenous peptides comprise cyanogen-bromide cleaved ovalbumin peptides.



6. A method according to claim 1, wherein the exogenous peptide is an immunogenic epitope for a pathogen.

7. A method of priming class I MHC-restricted cytotoxic T lymphocyte response with exogenous peptides comprising the steps of

- 5 (a) obtaining the exogenous peptides and  
(b) presenting exogenous peptides to antigen presenting cells bearing class I MHC molecules in the presence of an amount of free  $\beta_2$ -microglobulin and culturing the thus-prepared cells in the presence of a naive population of T lymphocytes  
10 wherein the  $\beta_2$ -microglobulin is present in an amount sufficient to stimulate an immune response.

8. A method according to claim 7 wherein the  $\beta_2$ -microglobulin is selected from the group consisting of purified human  $\beta_2$ -microglobulin, purified murine  $\beta_2$ -microglobulin, purified bovine  
5  $\beta_2$ -microglobulin and purified equine  $\beta_2$ -microglobulin and mixtures thereof.

9. A method according to claim 8, wherein the exogenous peptides are presented to antigen presenting cells bearing class I MHC molecules in the presence of an elevated amount of free  $\beta_2$ -  
5 microglobulin in vitro.

10. A method according to claim 8, wherein the exogenous peptides are presented to antigen presenting cells bearing class I MHC molecules in the presence of an elevated amount of free  $\beta_2$ -microglobulin in vivo.

11. A method of using purified  $\beta_2$ -microglobulin as an adjuvant for a synthetic peptide vaccine comprising the steps of: obtaining purified  $\beta_2$ -microglobulin and presenting synthetic polypeptides comprising pathogen-derived epitopes to mammalian host immune cells in the presence of elevated levels of free  $\beta_2$ -microglobulin wherein the  $\beta_2$ -microglobulin is present in an amount sufficient to stimulate an immune response.

12. A method according to claim 11, wherein the synthetic peptides and free  $\beta_2$ -microglobulin are simultaneously injected into the mammalian host.

13. A method according to claim 11, wherein the synthetic peptides and free  $\beta_2$ -microglobulin are sequentially injected into the mammalian host.

14. A method of inducing class I MHC-restricted cytotoxic T lymphocyte response to a pathogen-related immunogen in a mammalian host comprising the steps of

(a) obtaining a pathogen-related immunogen and

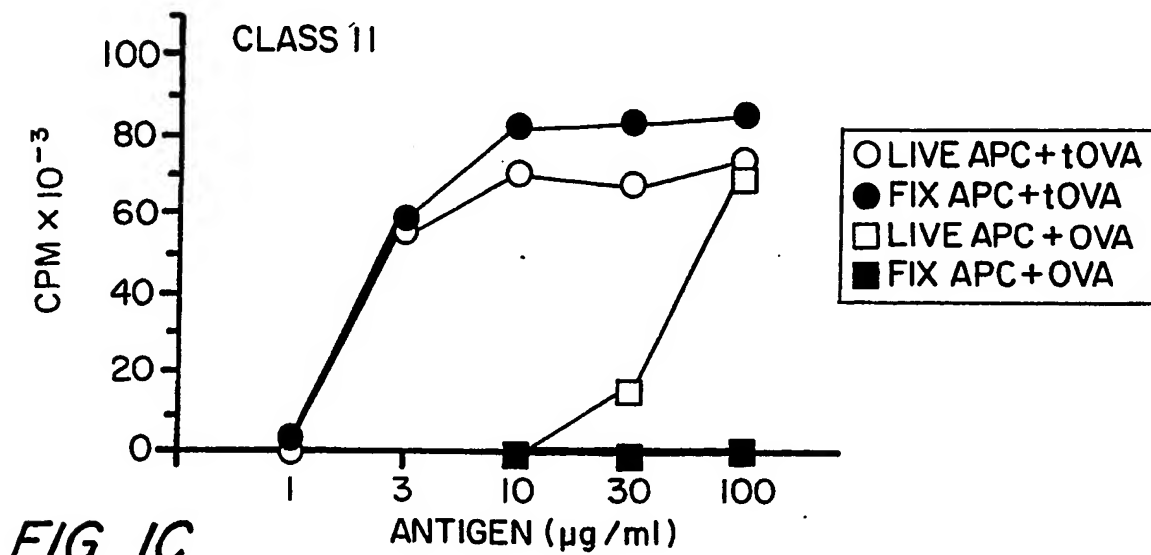
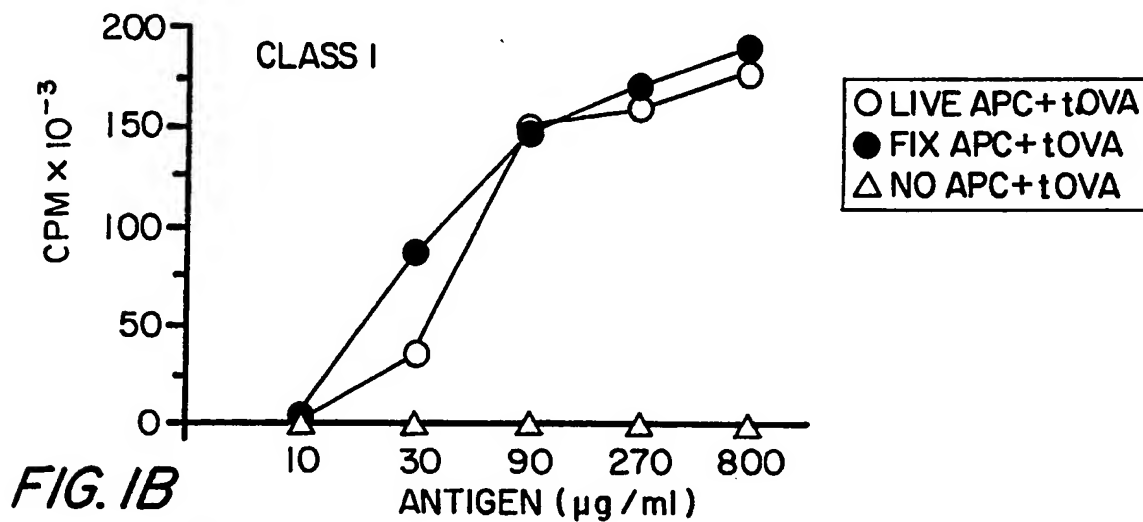
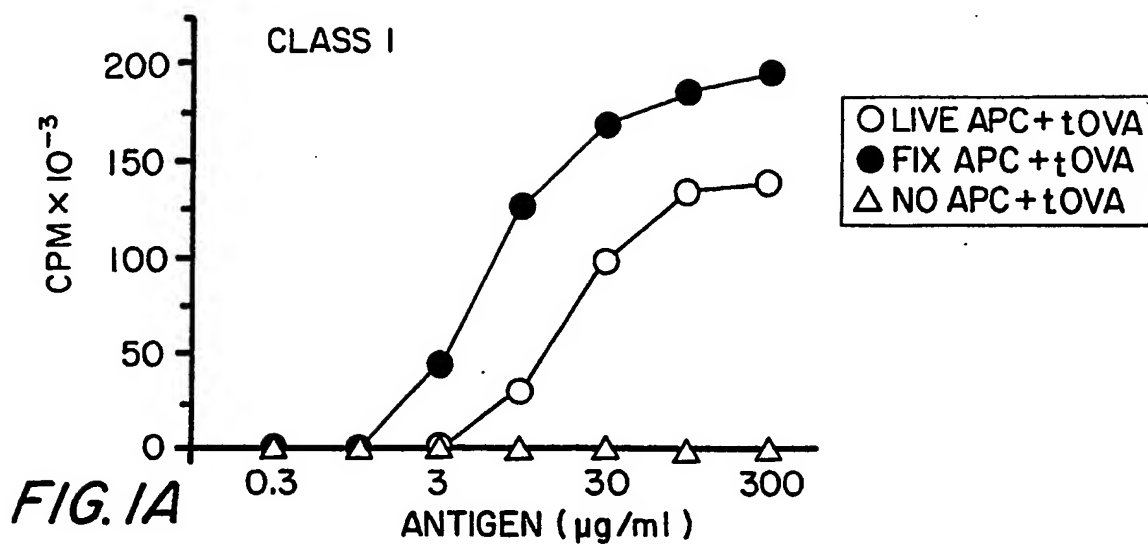
(b) exposing a naive population of T lymphocytes to the pathogen-related immunogen in the presence of free  $\beta_2$ -microglobulin, said  $\beta_2$ -microglobulin being present in a concentration  
5 greater than the concentration of  $\beta_2$ -microglobulin normally present in mammalian serum and sufficient to cause an immune response.

15. A method according to claim 14, wherein the  $\beta_2$ -microglobulin is selected from the group consisting of purified human  $\beta_2$ -microglobulin, purified murine  $\beta_2$ -microglobulin, purified bovine  
5  $\beta_2$ -microglobulin, purified equine  $\beta_2$ -microglobulin and mixtures thereof.

16. A method according to claim 15, wherein the  $\beta_2$ -microglobulin is purified human  $\beta_2$ -microglobulin.

17. A method according to claim 16, wherein the free  $\beta_2$ -microglobulin is introduced into the mammalian host in an amount that is from about 2 to about 10 fold higher than that normally present in  
5 the serum of said mammalian host.

1/8



2/8

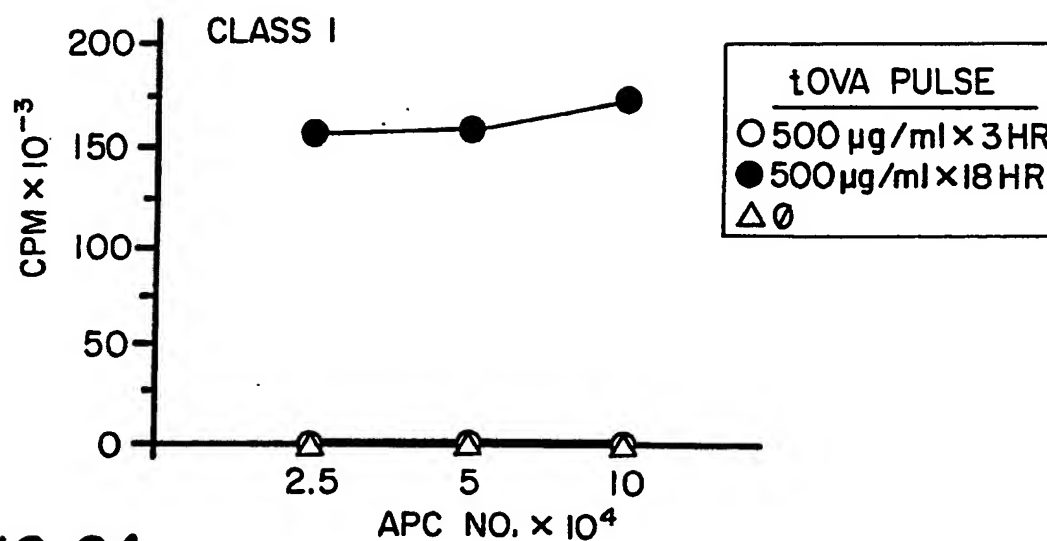


FIG. 2A

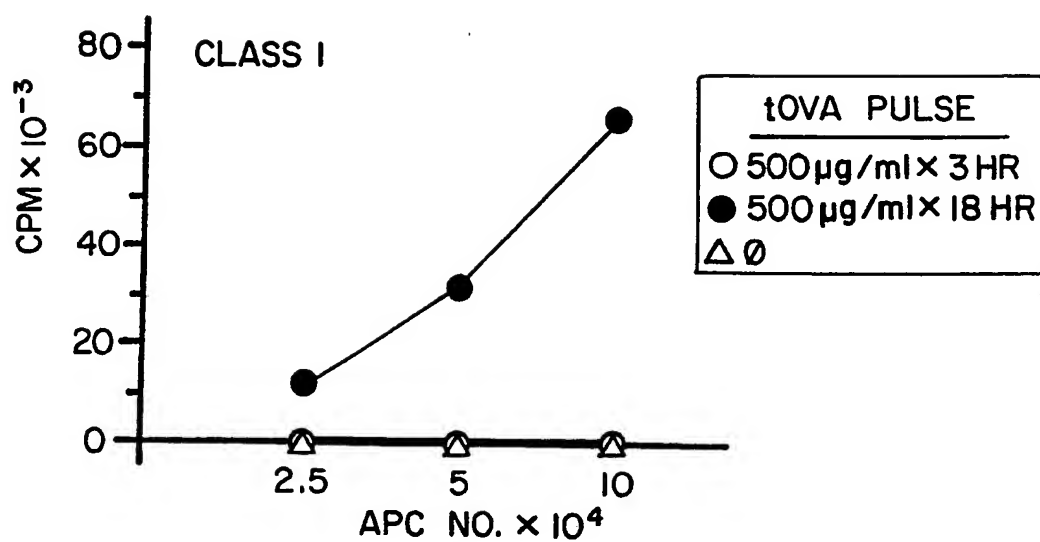


FIG. 2B

3/8

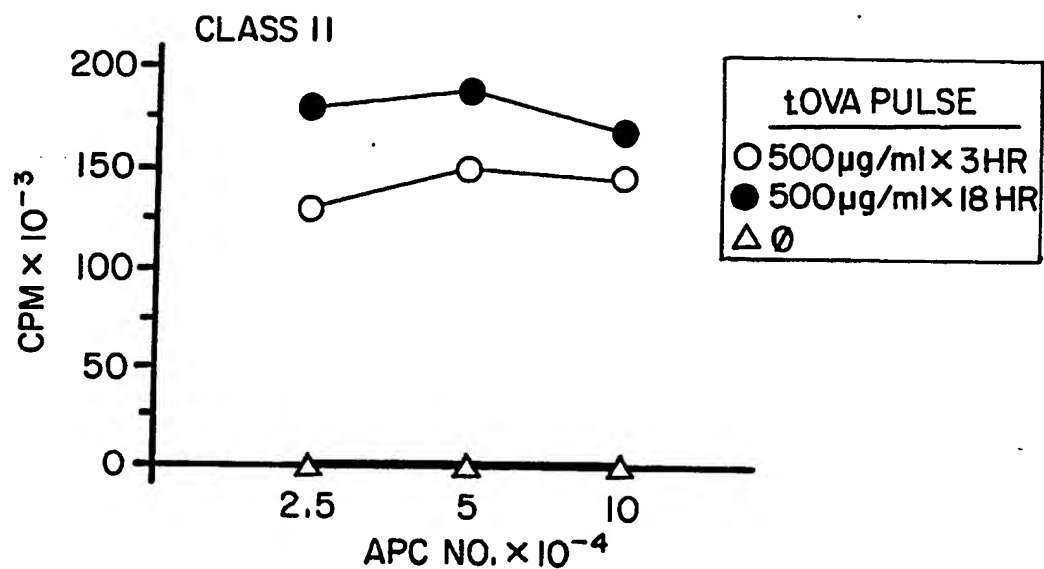


FIG. 2C

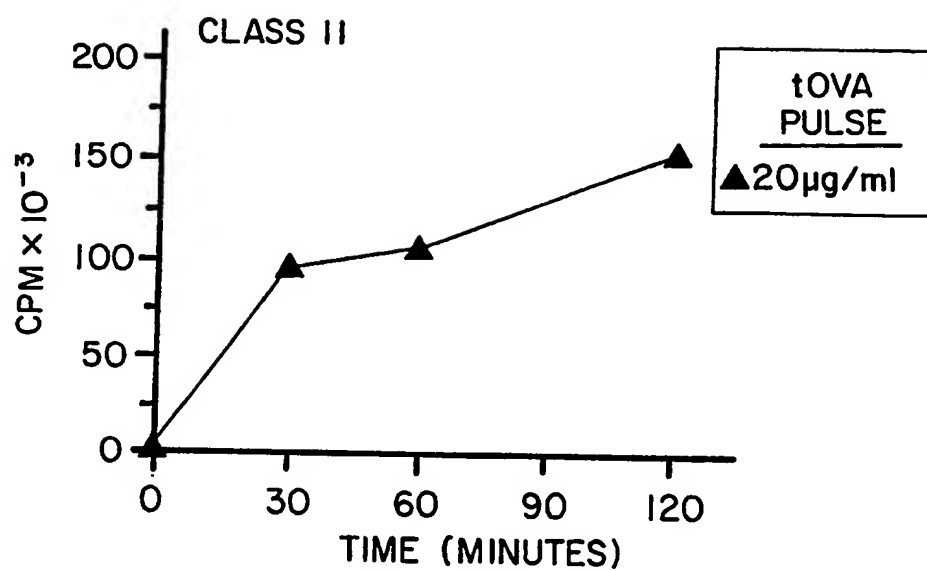
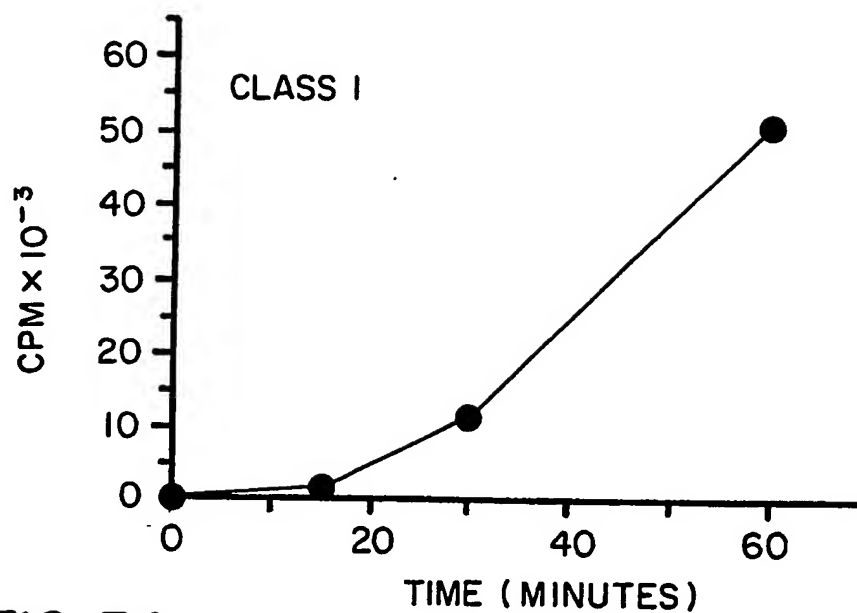
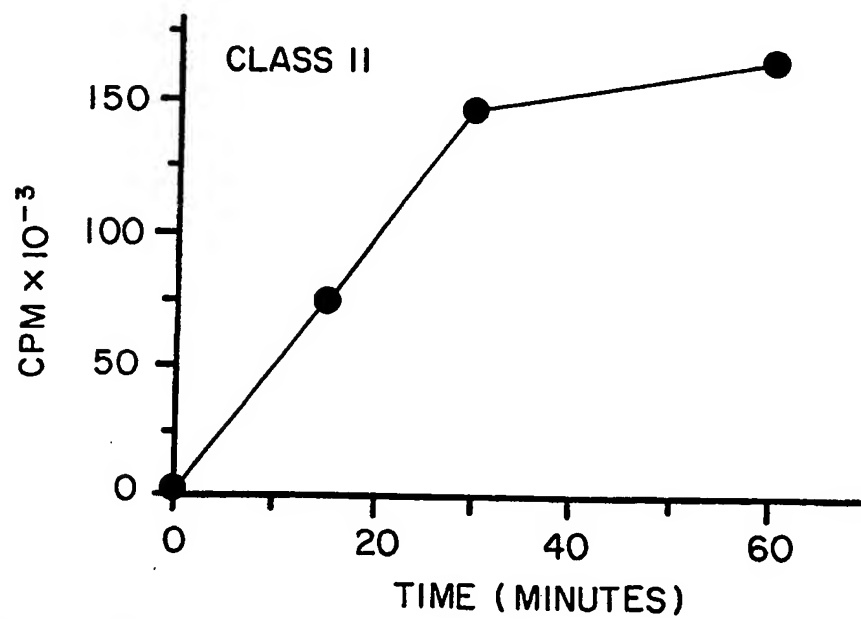
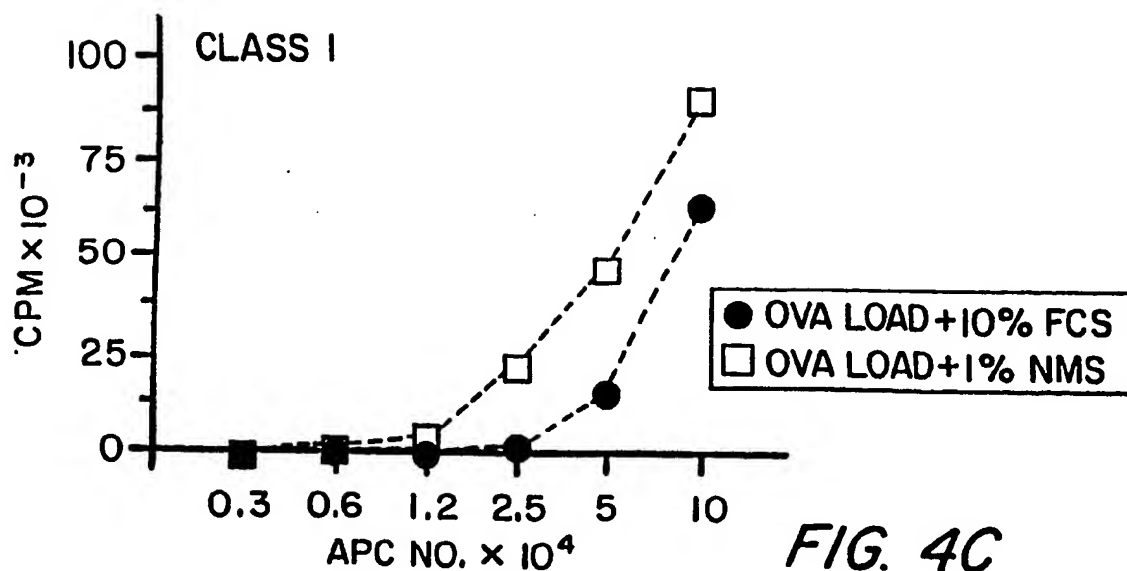
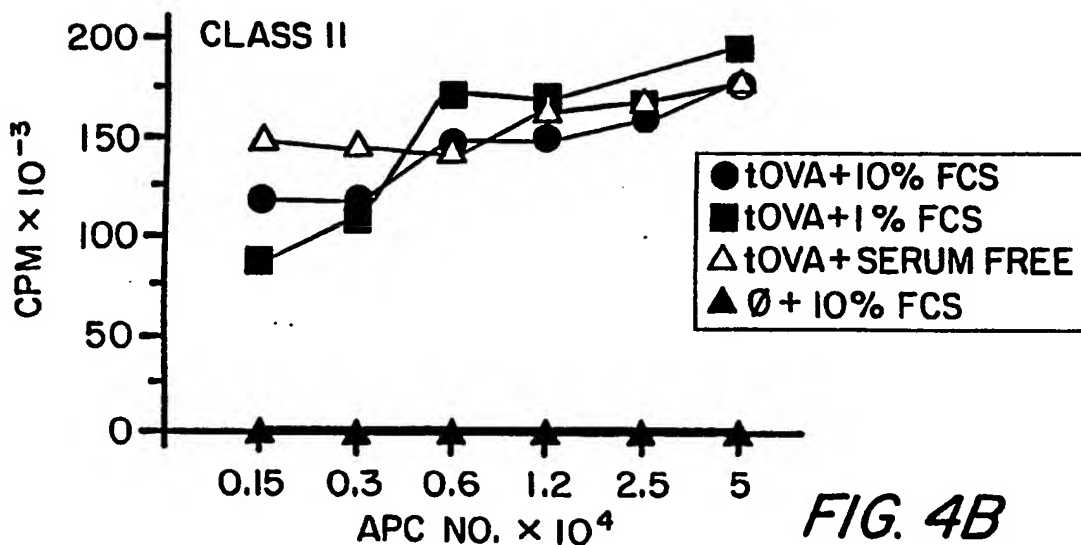
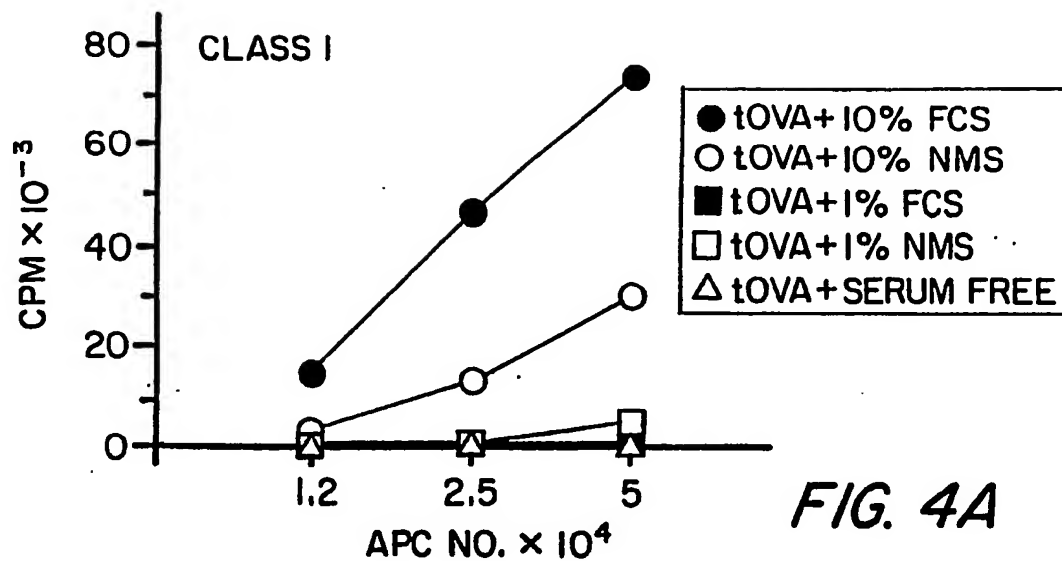


FIG. 2D

4/8

*FIG. 3A**FIG. 3B*

- 5 / 8 -





6/8

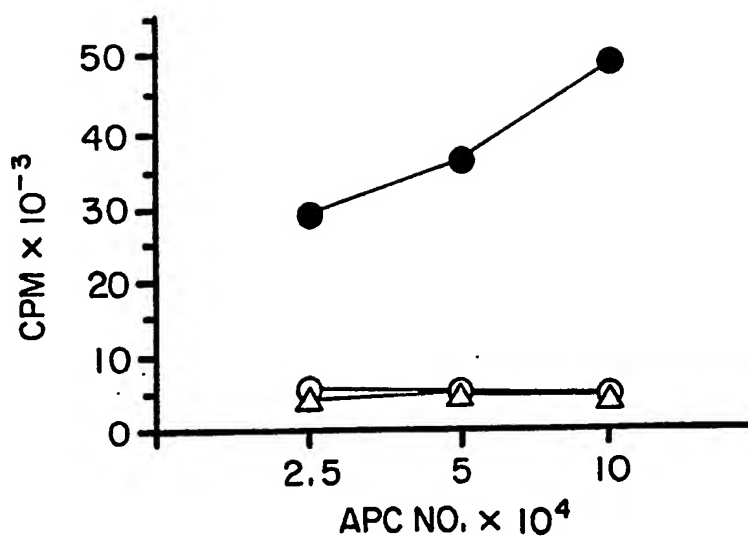


FIG. 5A

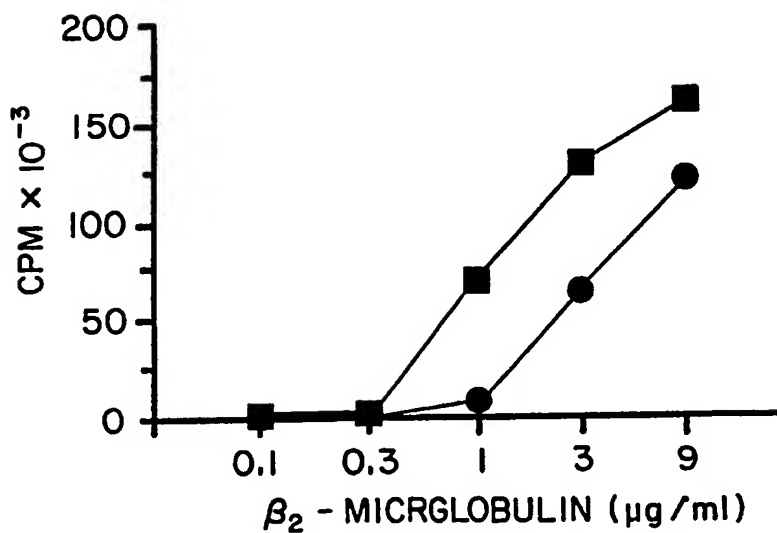


FIG. 5B

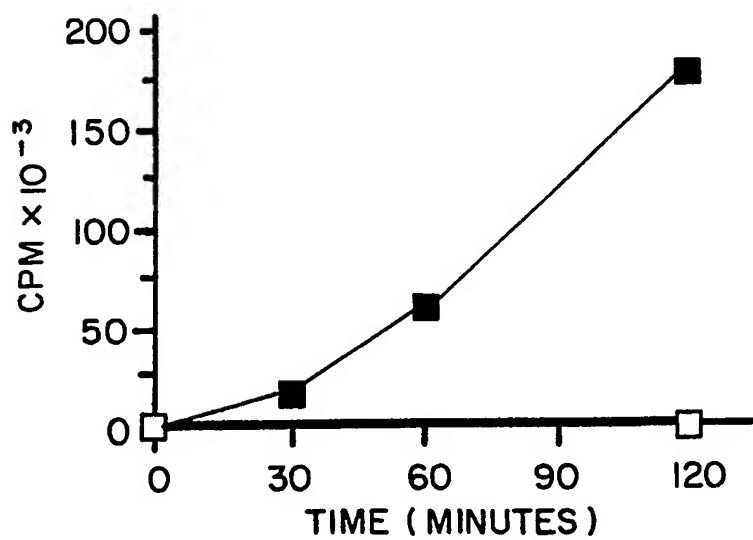


FIG. 5C

7/8

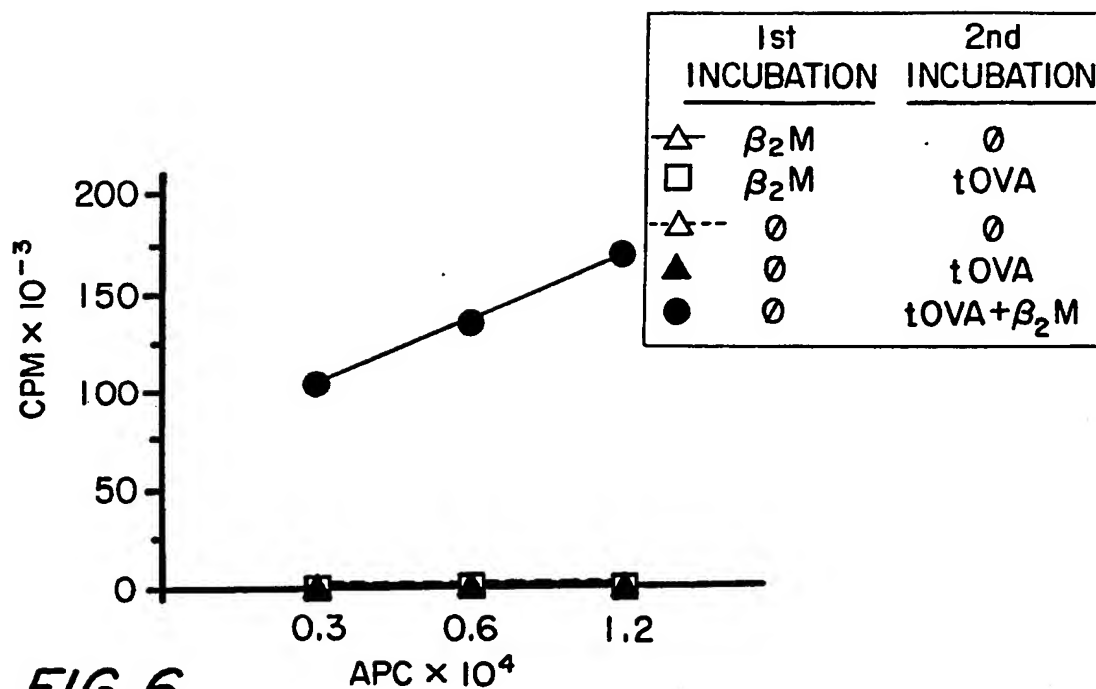


FIG. 6

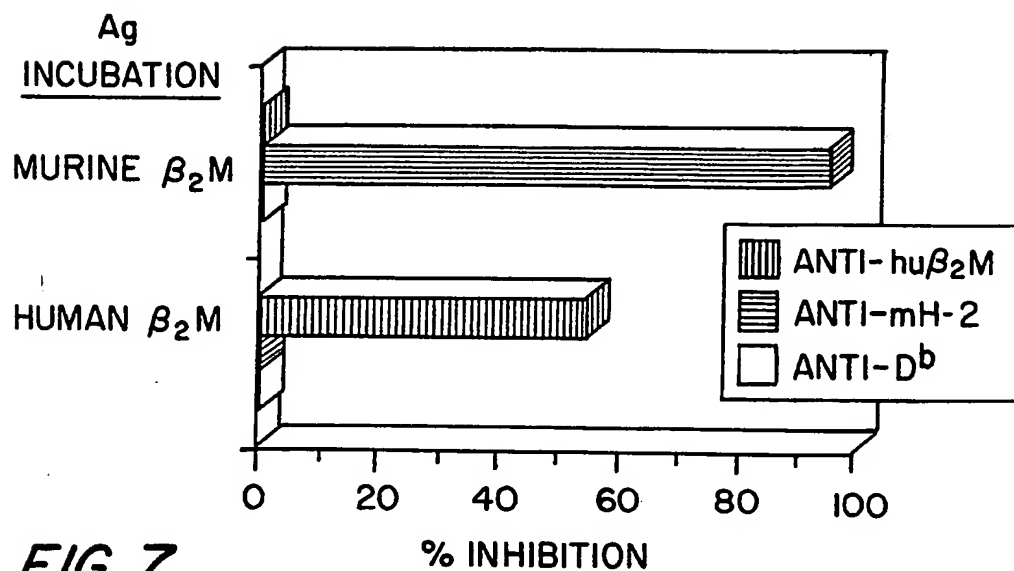
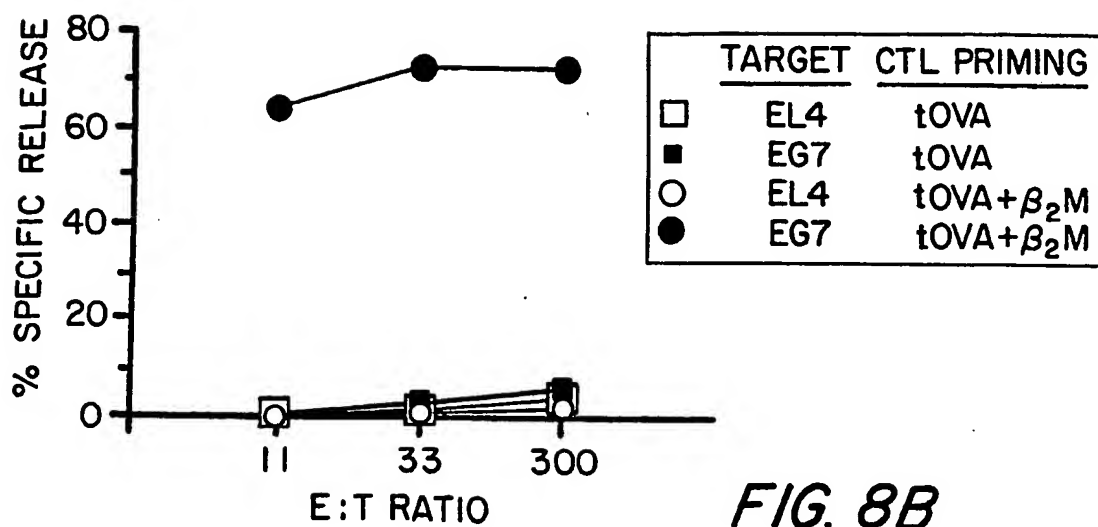
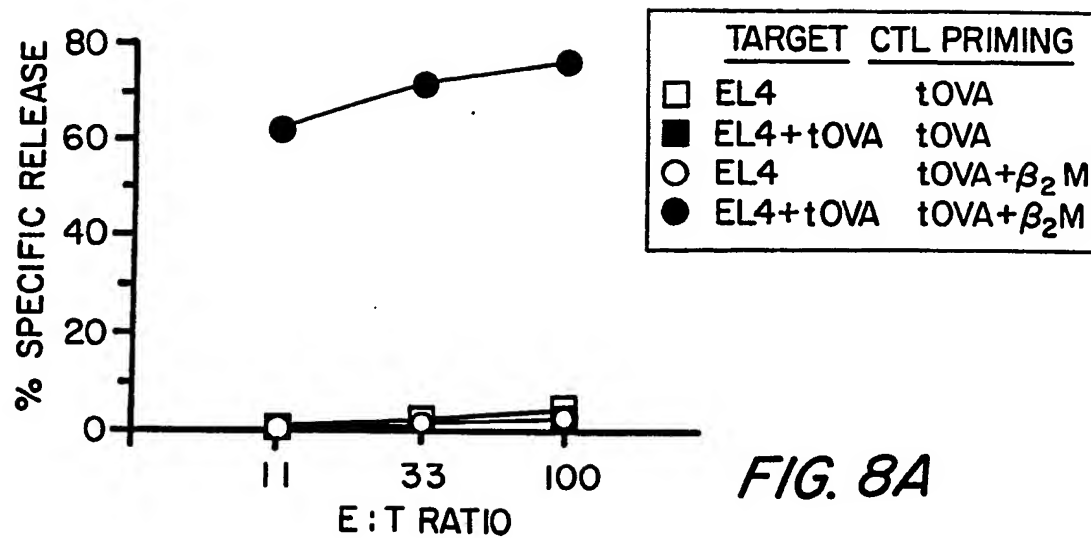


FIG. 7

8/8



# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US91/03082**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate)		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5): A61K 39/00, 37/00, 49/00 U.S. Cl: 424/88, 435/240.1; 514/8, 21		
<b>II. FIELDS SEARCHED</b>		
Classification System	Minimum Documentation Searched	Classification Symbols
U.S.	424/88; 435/240.1; 514/8, 21	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched		
Databases: Dailog (files 5, 399, 154, 357); USPTO Automated Patent System (File USPAT, 1971-1991).		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No. 1
Y	Nature, Volume 308, issued 12 April 1984, Bernabeu et al., B <sub>2</sub> -Microglobulin from Serum Associates with MHC class I antigens on the surface of cultured cells", pages 642-645, see abstract.	1-4
Y	Science, Volume 239, issued 05 February 1988, Yewdell et al., "Cells process exogenous proteins for recognition by cytotoxic T lymphocytes", pages 637-640, see entire article.	1-10
Y	Proceedings of the National Academy of Sciences, Volume 76, No. 11, issued November 1979, Hyatil et al., "Dissociation and exchange of B <sub>2</sub> Microglobulin subunit of HLA-A and HLA-B antigens", pages 5834-5838, see entire document.	1-17
Y	Cell, volume 54, issued 09 September 1988, Moore et al., "Introduction of Soluble Protein into the class I Pathway of Antigen Processing and Presentation", pages 777-785, see entire document.	1-17
Y	Journal of Experimental Medicine, volume 167, issued June 1988, Carbone et al., "Induction of Cytotoxic	14-17
(See Attachment)		
* Special categories of cited documents:		
"A" document defining the general state of the art which is not considered to be of particular relevance		
"E" earlier document but published on or after the international filing date		
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"O" document referring to an oral disclosure, use, exhibition or other means		
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention		
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step		
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.		
"A" document member of the same patent family		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		
27 June 1991		
International Searching Authority		
ISA/US		
Date of Mailing of this International Search Report		
03 SEP 1991		
Signature of Authorized Officer		
Lynette F. Smith		
bb		

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (In several classification systems, such as IPC)		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5): A61K 39/00, 37/00, 49/00 U.S. Cl.: 424/88, 435/240.1: 514/8, 21		
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Classification System	Minimum Documentation Searched	Classification Symbols
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Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched		
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Y	Cell, volume 54, issued 09 September 1988, Moore et al., "Introduction of Soluble Protein into the class I Pathway of Antigen Processing and Presentation", pages 777-785, see entire document.	1-17
Y	Journal of Experimental Medicine, volume 167, issued June 1988, Carbone et al., "Induction of Cytotoxic (See Attachment)	14-17
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"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.		
"Δ" document member of the same patent family		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
27 June 1991		03 SEP 1991
International Searching Authority		Signature of Authorized Officer
ISA/US		Lynette F. Smith

bb

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (Inventor's classification)	
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): A61K 39/00, 37/00, 49/00 U.S. Cl.: 424/88, 435/240.1; 514/8, 21	
<b>II. FIELDS SEARCHED</b>	
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
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Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 June 1991	03 SEP 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	Lynette F. Smith

## III. DOCUMENTS CONSIDERED

BE RELEVANT

(CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, 1 <sup>st</sup> with indication, where appropriate, of the relevant passages 1 <sup>st</sup>	Relevant to Claim No 1 <sup>st</sup>
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T lymphocytes By Primary In Vitro Stimulation With Peptides" pages 1767-1779, see entire document.